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A Comparative Study on the Role of Cytokinins in Caryopsis Development in the Maize *miniature1* Seed Mutant and Its Wild Type

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Abstract

We report here on a comparative developmental profile of plant hormone cytokinins in relation to cell size, cell number and endoreduplication in developing maize caryopsis of a cell wall invertase-deficient *miniature1* (*mn1*) seed mutant and its wild type, *Mn1*, genotype. Both genotypes showed extremely high levels of total cytokinins during the very early stages of development, followed by a marked and genotype specific reduction. While the decrease of cytokinins in *Mn1* was associated with their deactivation by 9-glucosylation, the absolute and the relative part of active cytokinin forms was higher in the mutant. During the exponential growth phase of endosperm between 6 d after pollination and 9 d after pollination, the mean cell doubling time, the absolute growth rate and the level of endoreduplication were similar in the two genotypes. However, the entire duration of growth was longer in *Mn1* compared with *mn1*, resulting in a significantly higher cell number in the *Mn1* endosperm. These data correlate with the previously reported peak levels of the *Mn1*-encoded cell wall invertase-2 (INCW2) at 12 d after pollination in the *Mn1* endosperm. A model showing possible crosstalk among cytokinins, cell cycle and cell wall invertase as causal to increased cell number and sink strength of the *Mn1* developing endosperm is discussed.

Key words: 9-glucosylation; cell wall invertase; cytokinins; maize caryopsis; *miniature1*.

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In the caryopsis, the unique fruit of plants from the grass family, a single seed contains an embryo surrounded by an endosperm. Following fertilization, the embryo and endosperm act as "utilization sinks" in which cell division activity governs sink strength, but at maturity, the endosperm constitutes a storage sink (Ho 1988). It then develops into a nutritive tissue used at germination. Molecular mechanisms involved in these processes are not well understood. However, regulation of cell divisions and sink strength requires a network that links various individual signals at different regulatory levels. The hydrolytic enzyme, cell wall invertase (CWI) and plant hormone cytokinins are likely participants in this system. Cytokinins play a crucial role in regulating proliferation and differentiation of plant cells, and also control various processes in plant growth and development (reviewed in Sakakibara 2006). CWI localized in the apoplast and ionically bound to the cell wall catalyses the irreversible cleavage of sucrose. The resulting hexoses are then imported into sink cells by hexose transporters and are crucial in establishing sink strength. Notably, in cultured cells of *Chenopodium rubrum*, the expression of CWI and of plasma membrane hexose uptake receptors is enhanced by cytokinins. The latter could also alter the source–sink balance in *SAG12-IPT* plants by inducing extracellular invertase (reviewed in Roitsch and Gonzáles 2004). The upregulation of CWI by cytokinins has a dual function, providing carbohydrates to the actively growing tissues and generating a metabolic signal

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to stimulate the cell cycle. This mechanism ensures that cell proliferation is only initiated when sufficient carbohydrates are available to satisfy the increased demand for nutrients by the actively dividing cells (reviewed in Roitsch and Gonzáles 2004).

The aim of the work reported here was to analyze cytokinin metabolites in the *miniature1* (*mn1*) maize seed mutant to evaluate their possible contribution to its development. *mn1* shows a drastically smaller endosperm size than that of the wild type, *Mn1*, with the weight of the mature *mn1* endosperm being only 30% that of *Mn1* (Lowe and Nelson 1946). Its smaller size at 16 d after pollination (DAP) is associated with a reduced mitotic activity in the developing endosperm (Vilhar et al. 2002). The causal basis of the *mn1* seed phenotype is the loss of one of the two CWI isoforms, INCW2, encoded by the *Mn1* gene, which is specifically expressed at the base of the endosperm (Cheng et al. 1996). The *mn1* mutation is associated with loss of ~98% of the invertase enzyme activity. The residual invertase activity in the *mn1* mutant is encoded by the *Incw1* gene (Chourey et al. 2006). Although cytokinins have been studied in maize caryopsis in recent decades (Dietrich et al. 1995; Bilyeu et al. 2001; Brugière et al. 2003), we have re-examined them together with a cytological analysis of the early caryopsis development to examine the possible crosstalk of cytokinins with CWI and the cell cycle.

Results

Cytological analysis of caryopsis development

At 4 DAP the endosperms of *Mn1* and *mn1* had similar numbers of cells (Figures 1, 2). Endosperms were small and embedded in the prevailing maternal nucellar tissue (Figure 1).

The increasing absolute growth rate between 6 and 12 DAP, with its peak at 12 DAP in *Mn1* (Figure 3), resulted in a steep increase in cell number, both in the endosperm and in the embryo (Figures 1, 2). The absolute growth rate in *mn1* slowed down around 9 DAP (Figure 3) and, as a consequence, the cell number of *mn1* endosperm started to lag behind that in *Mn1* (Figure 2). After 8 DAP the mean cell doubling time progressively lengthened in *mn1* and was 1.79-fold longer than in *Mn1* in the interval between 10 and 12 DAP (Figure 3). Between 8 and 12 DAP most of the daughter cells maintained the initial 3C/6C nuclear DNA content (Figure 1) and were of the same size in both genotypes (Figure 4). However, at 8 DAP a few nuclei were already endopolyploid in both endosperms and, at 12 DAP, some large cells with highly endoreduplicated nuclear DNA (up to 192C) were detected (Figure 1).

Cytokinin analysis

At 6 DAP the total cytokinin concentrations were 16 and 12.2 nmol/g DW and, at 8 DAP, 13.1 and 15.3 nmol/g DW in the *Mn1* and *mn1* caryopsis, respectively (Figure 2). Notably, at 8 DAP there was 1.8-times more cytokinins in the *Mn1* basal section than in the upper one (Figure 5), while the same ratio in the *mn1* caryopsis was 1.3 (Figure 5). The very high concentration of cytokinins in the endosperms of both genotypes at 6–8 DAP dropped substantially at ~12 DAP and beyond (Figures 2, 5). However, their total concentration in *Mn1* decreased nine-fold between 8 and 12 DAP, compared with less than five-fold in *mn1* (Figure 2). The extent of the decrease in cytokinin concentration was more pronounced in the upper section of the *Mn1* caryopsis (16-fold), compared with the basal section (seven-fold) (Figure 5). On the other hand, the cytokinin concentration decrease from 8 to 12 DAP was similar in the basal (five-fold) and upper (four-fold) sections of the *mn1* caryopsis (Figure 5). As a result, the difference in the cytokinin concentrations in the upper section of the wild type and mutant caryopsis was highly significant at 12 DAP (Figure 5). The diminished cytokinin concentration in *Mn1* correlated with a diminished diversity of cytokinin isoforms. After 12 DAP mostly zeatin type cytokinins were detected in *Mn1*, while in *mn1* most of the cytokinins identified at earlier stages were still present (Figure 5).

Two free bases (*trans*-zeatin, Z; dihydrozeatin, DHZ), three ribosides (*trans*-zeatin riboside, ZR; dihydrozeatin riboside, DHZR; N^6-isopentenyl adenosine, iPA) and one 9-glucoside form (*trans*-zeatin-9-glucoside, Z-9-G) of cytokinins were detected in each of the genotypes. However, their distribution pattern changed differently in *Mn1* and *mn1* with time (Figure 5).

The main cytokinin in the *Mn1* caryopsis at 6 DAP was Z and it comprised 57% of all detected cytokinins (i.e. 9170 pmol/g DW). Although up to 20 DAP Z was the most abundant cytokinin metabolite in the basal section (Figure 5), at 12 and 16 DAP its concentration was similar to that of ZR and was almost two times higher than that of ZR at 20 DAP. On the other hand, in the upper section at 8 DAP, Z concentration was similar to the concentration of ZR, but was substantially lower afterward (Figure 5).

In the mutant caryopsis the level of Z remained high from 8 to 20 DAP in both the basal and upper section. While Z was the main cytokinin in the basal section throughout development, it exchanged this position with ZR in the upper section at 12 DAP (Figure 5).

Dihydrozeatin and dihydrozeatin riboside were abundant cytokinin forms at 8 DAP in the caryopsis of both genotypes (Figure 5). Although they were on or below the detection limit in the *Mn1* caryopsis at 12 DAP and afterward, they were still detectable in the *mn1* caryopsis at later stages (Figure 5).

N^6-isopentenyl adenosine that was detected at 8 DAP in *Mn1* and *mn1* was below the detection limit in the wild type caryopsis after 12 DAP. Nevertheless, iPA was still present in the *mn1* caryopsis after 12 DAP and in their upper sections iPA levels showed two peaks: one at 8 DAP and the second one at 20 DAP (Figure 5).

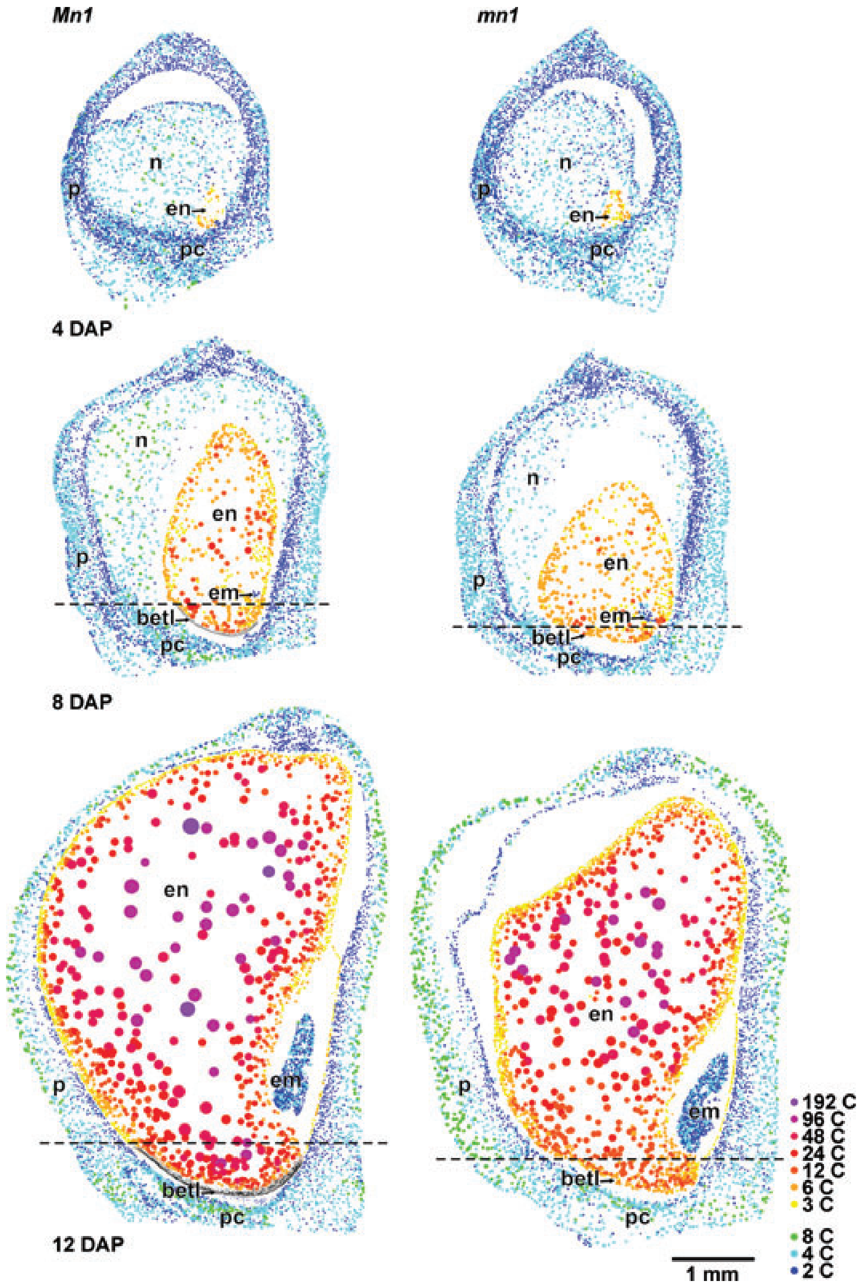

Figure 1. Developing *Mn1* and *mn1* maize caryopsis at 4, 8 and 12 d after pollination (DAP).

Images demonstrate the increase in cell number and the process of endoreduplication. Instead of entire cells, the actual nuclei in the caryopsis sections are shown as bubble graphs, where the different endopolyploidy classes are shown in different colors and the diameters of bubbles are linearly related to the diameters of nuclei. The DNA content of nuclei was measured *in situ* in median longitudinal tissue sections and shown as the C-value. The dotted lines indicate the site of caryopsis cutting to the basal and upper section before cytokinin analysis. betl, basal endosperm transfer layer; em, embryo; en, endosperm; n, nucellus; p, pericarp; pc, P-C layer.

Between 12 and 20 DAP, Z-9-G constituted an important proportion of cytokinins in caryopsis. Although the absolute concentration of Z-9-G did not change significantly with time, its relative concentration steadily increased due to the diminishing concentration of other cytokinins (Figure 5). In the period between 12 and 20 DAP, 17–31% of the total cytokinins detected in *Mn1* were in the Z-9-glucolyzed form, while in *mn1* over the same period, this metabolite constituted only a small fraction

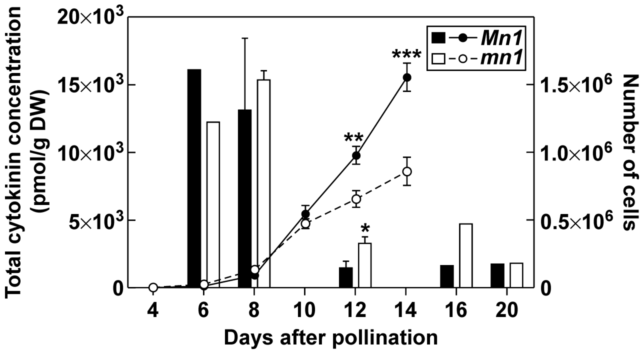

Figure 2. Total cytokinin concentration and the number of endosperm cells in *Mn1* and *mn1* caryopsis.

Concentration results (columns) of 6, 16 and 20 d after pollination (DAP) charyopses are from one growing season experiment, and those of 8 and 12 DAP are the means ± *SE* of two to three samples from each of two different growing seasons. Endosperm cell numbers (lines) are the means ± *SE* of three to four caryopses per developmental stage from one growing season. Student's *t*-test between *Mn1* and *mn1* data, $^{*}P < 0.1$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of the total (Figure 6). In the upper parts of caryopsis, at 12 DAP, 9-glucosylation was significantly higher in *Mn1* than in *mn1* (Figure 6). At 20 DAP, 91% of cytokinins in the upper parts of the *mn1* caryopsis were still in the forms of free bases and ribosides, but in the basal sections these moieties decreased to 66%, and thus were similar to those in *Mn1* (Figure 6).

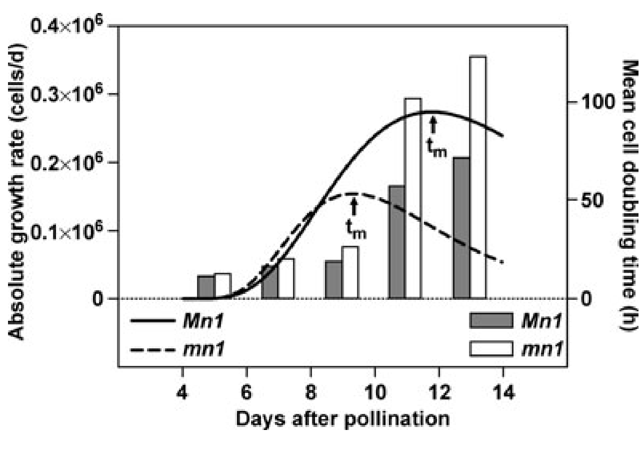

Figure 3. Absolute growth rate (lines) and mean cell doubling time (columns) in *Mn1* and *mn1* maize endosperm between 4 to 14 d after pollination (DAP), calculated from three to four caryopses per developmental stage from one growing season. t_m, the time when the absolute growth rate is maximal.

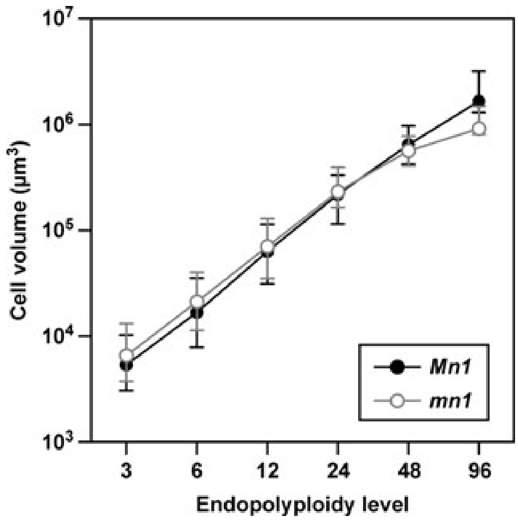

Figure 4. Relationship of cell volume to the endopolyploidy level in *Mn1* and *mn1* maize endosperm at 12 d after pollination (DAP).

Median cell volumes and interquartile range in the endosperm of one *Mn1* and one *mn1* 12 DAP caryopsis are shown on the graph. Due to the small number of cells with an endopolyploidy level 192C, they were pooled together with 96C cells.

Discussion

Cytokinin distribution

The cytokinin metabolites observed here in the *Mn1* and *mn1* caryopsis of the W22 inbred background are the same as those reported previously in the inbred line B73 (Brugière et al. 2003; Veach et al. 2003). In addition, we show here for the first time the presence of DHZ. Considering the very low recovery of N^6-isopentenyl adenine (iP), the presence of this cytokinin metabolite might be below the detection limit of our system. Similarly, it has not been reported in other studies of cytokinins in maize caryopsis. *Cis*-isomers of Z- or ZR-type cytokinins, which were shown by Veach et al. (2003) were not detected by the applied system, but their occurrence could not be ruled out.

The most abundant cytokinin metabolite in the caryopsis of *Mn1* and *mn1* was Z (Figure 5). However, its spatial and temporal distribution differed; thus Z was on or even below the detection limit in the upper sections of the wild type caryopsis between 12 and 20 DAP (Figure 5). On the contrary, in the caryopsis of the inbred line, B73 ZR prevailed up to 20 DAP in the basal and upper sections (Brugière et al. 2003). Although the role of the specific cytokinin metabolite is unclear, it has been suggested that ZR and Z regulate both carbohydrate and protein metabolism. However, ZR primarily affects the metabolism of carbohydrate reserves and Z the protein metabolism (Villalobos and Martin 1992). Currently, it is not known if different

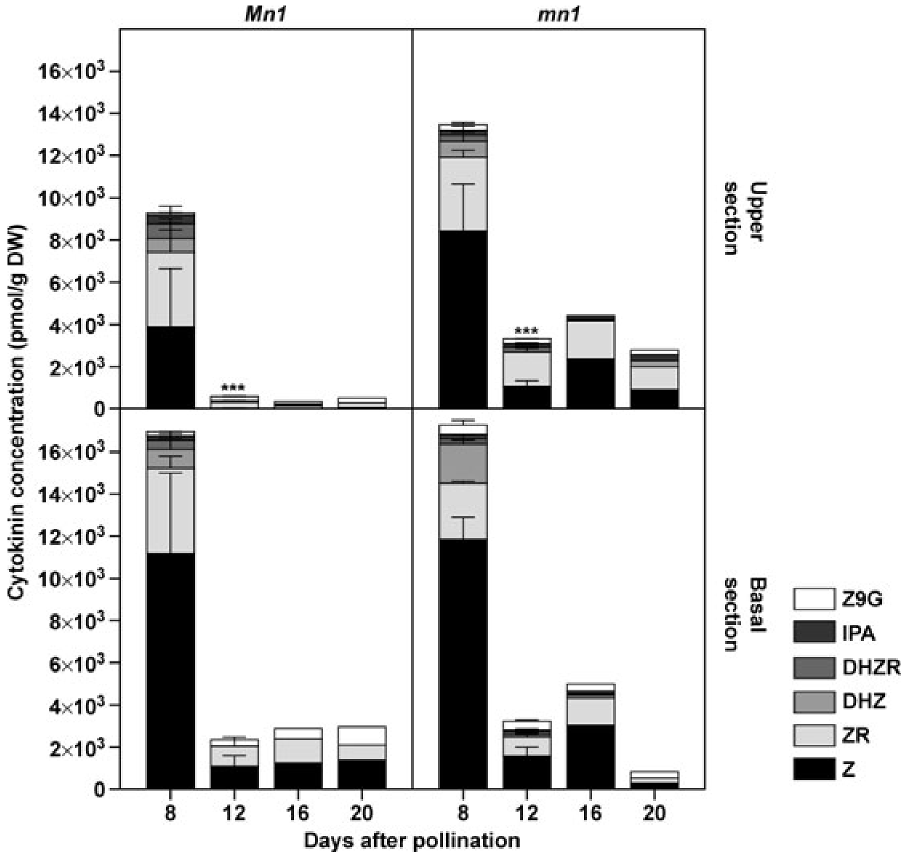

Figure 5. Distribution and concentration of cytokinins at 8, 12, 16 and 20 d after pollination (DAP) in the basal and upper sections of *Mn1* and *mn1* caryopsis.

Concentrations of 8 and 12 DAP caryopsis are the means ± *SE* of two to three samples from each of two different growing seasons. Student's *t*-test between *Mn1* and *mn1* 12 DAP upper section. $^{***}P < 0.001$. Concentrations of 16 and 20 DAP caryopsis are from one growing season.

distributions of Z and ZR in the maize caryopsis are associated with the previous observation that signals, which affect cell division and elongation are independent from those which control the storage function of starch biosynthesis (Cheng and Chourey 1999).

Cytokinin concentrations were generally higher in the basal section of caryopsis than in the upper one (Figure 5), in agreement with Brugière et al. (2003). The peak of cytokinins in the pedicel/chalazal region (Figure 5) coincides with the maximum expression of *ZmIPT2* and its encoded gene cytokinin biosynthetic enzyme ZmIPT2 in the basal endosperm transfer cells (Brugière et al. 2008). This finding is consistent with the hypothesis of *ZmIPT2* as a cytokinin biosynthetic gene, which might enable cytokinin biosynthesis in the caryopsis. In favor of this idea are also our previous experiments with *in vitro* maize kernel culture, which thrives in cytokinin free media (Cheng and Chourey 1999). Currently, there is no experimental evidence for the alternative possibility that cytokinins are transported from the maternal tissue to the developing caryopsis via xylem sap, such as the main cytokinin transported system in maize (Takei et al. 2001).

The cytokinin peak preceded the developmental phase with the highest caryopsis growth rate (Figures 1–3). Based on the correlative literature data between cell divisions (Kowles and Phillips 1985) and high cytokinin levels soon after pollination (Dietrich et al. 1995; Brugière et al. 2008) the role of cytokinins in active endosperm cell divisions has been suggested (Dietrich et al. 1995; Brugière et al. 2008). However, our precise measurements of cell divisions (Figures 1–3) and cytokinins in the corresponding caryopsis (Figures 2, 5) did not show strong agreement. It is noteworthy that the cytokinin peak appeared well before the peak of cell divisions (Figures 1–3, 5), which might indicate an additional physiological role for cytokinins in the early days after pollination.

At around 12 DAP, a substantial drop in the concentration of free bases (Z, DHZ) and ribosides (ZR, DHZR, iPA) was detected in the caryopsis of both genotypes (Figures 2, 5), but the decrease was particularly emphasized in *Mn1*. It is

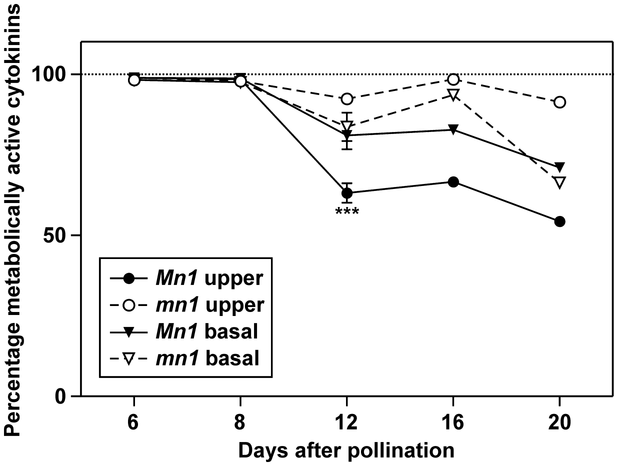

Figure 6. Metabolically active cytokinins (free bases and ribosides) expressed as a percentage of total cytokinin concentration in the basal and upper sections of *Mn1* and *mn1* caryopsis.

Concentration results of 6, 16 and 20 d after pollination (DAP) charyopses are from one growing season experiment, and those of 8 and 12 DAP are the means $\pm$ *SE* of two to three samples from each of two different growing seasons. Student's *t*-test between *Mn1* and *mn1* 12 DAP upper section. $^{***}P < 0.001$.

generally held that cytokinin oxidase (Ckx) is the primary means of irreversible cytokinin degradation by cleaving the N^6-side chain from the adenine/adenosine moiety and thus provides an important mechanism by which plants modulate their cytokinin level. This role of Ckx is also evidenced in maize caryopsis by its high activity levels at 11 DAP and beyond (Brugière et al. 2003). At 12 DAP and later, the level of dihydrozeatin type metabolites was mostly undetectable in *Mn1* caryopsis (Figure 5). This is noteworthy, since DHZ is resistant to Ckx, since the enzyme does not recognize the double bond of the isoprenoid side chain by the enzyme (Mok and Mok, 2001). Therefore, the transformation of dihydrozeatin type cytokinins to other metabolites that are not detected by the applied immunochromatographic system cannot be excluded.

The cytokinin composition in the period between 12 and 20 DAP in *Mn1* was characterized by a notably high concentration of Z-9-G (Figures 5, 6). Interestingly, Z-9-G represented a significantly larger fraction in the upper *Mn1* caryopsis at 12 DAP than in *mn1*. At that point in time, most of the upper part of the caryopsis comprised endosperm (Figure 1). In addition to degradation with Ckx, the steady-state levels of active cytokinins *in planta* may also be regulated by a glucosylation at the N3, N7, or N9 position of the cytokinin purine moiety or at the hydroxyl group. Because N-glucosides are not cleaved efficiently by β-glucosidase, N-glucosylation is a practically irreversible process and thus 9-glucoside forms of cytokinins may be considered as metabolically inactive (reviewed in Sakakibara 2006).

Cell divisions between 8 and 12 DAP (Figures 1, 2) established the basic cell number for the body of the endosperm and may be important for grain yield (Kowles and Phillips 1985). The exponential growth phase of endosperm indicated that the mean cell doubling time (MCDT) and the absolute growth rate were similar in *Mn1* and *mn1* (Figures 2, 3). Moreover, a high number of cells with an initial DNA content of 3C/6C (Figure 1) and their like size in both genotypes (Figure 4), indicated a normal mitotic cycle (Francis 2007). However, the growth phase was markedly longer in *Mn1* compared with the mutant (Figure 3). These data correlate with increasing INCW2 activity in the wild type endosperm (Cheng et al. 1996).

The first occurrence of endoreduplication as early as 6 DAP (data not shown) and its rate of increase was the same for both genotypes (Figure 1) (Vilhar et al. 2002). Endoreduplication is a variant of the cell cycle in which chromosomal DNA is replicated without intervening mitotic division, leading to endopolyploid nuclei with polytene chromosomes (Joubès and Chevalier 2000), and is a well conserved cellular feature of seed development in maize (Kowles and Phillips 1985; Vilhar et al. 2002). Endoreduplication can be affected by a number of physiological and environmental factors and phytohormones appear to play an important role in the induction of endoreduplication in the endosperm. It has been demonstrated that auxin specifically stimulates nuclear DNA endoreduplication in maize, and it was suggested that enhanced endoreduplication is due to a changed auxin/cytokinin ratio (Lur and Setter 1993). Indeed, our measurements of auxin concentrations in *Mn1* and *mn1* showed a correlation of the first auxin peak at 12 DAP (LeClere et al. 2008) with a peak of endoreduplication (Figure 1). Moreover, it has been demonstrated in cultured cells of tobacco that exogenous application of auxin and cytokinin maintains cell division, while elongation and concomitant DNA endoreduplication are induced and maintained in a medium containing only auxin (Quélo et al. 2002). In future work we shall examine whether inverse temporal and spatial distribution of cytokinins (this study) and auxins (LeClere et al. 2008) may contribute to the onset and continuation of endoreduplication as well as cell divisions in maize caryopsis.

A role for cytokinins and INCW2 in early caryopsis development

Using a cell culture system, Roitsch and Gonzáles (2004) proposed a model (a modified version is shown in Figure 7), showing how CWI could contribute to the sink strength and sink size which is regulated by cytokinins. We have adapted a revised model to explain to some extent the phenotypical and developmental features of the *mn1*. According to the model, CWI enhances the sink strength by increasing the flow of photosynthates to sink tissues, thereby linking the metabolic sugar signal to the regulation of the cell cycle via D-type cyclins

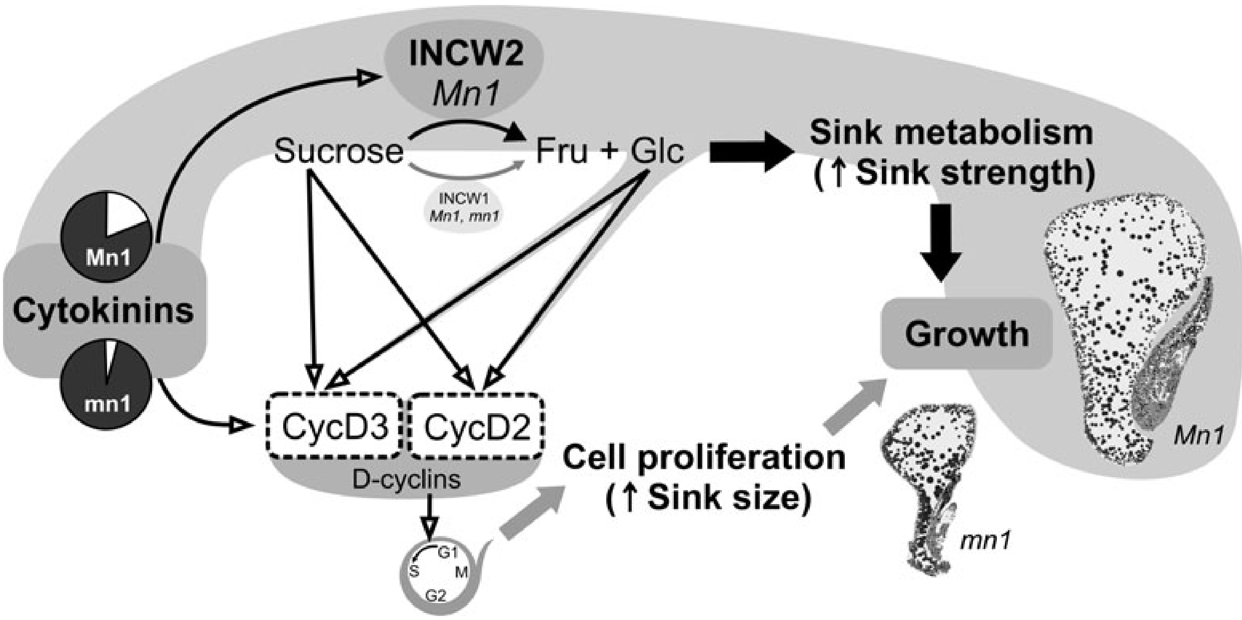

Figure 7. Model of two alternative developmental pathways induced by cytokinins that lead to different sizes of filial tissues (endosperm and embryo) in *Mn1* and *mn1* maize caryopsis.

Cytokinin pie charts indicate the ratio of metabolically active cytokinins (dark) versus metabolically inactive Z-9-G (white) in both genotypes. The pathway that uses INCW2 for increasing cell number and sink strength is shaded gray. The images of *Mn1* and *mn1* caryopsis are bubble graph representations of their actual longitudinal sections at 16 DAP. Steps in the model presented by dotted boxes and arrows with open heads have been proven in the experimental systems other than maize. Model adapted from Roitsch and Gonzáles (2004).

to increase sink size. Both, CWI and D-cyclins are stimulated by cytokinins (Riou-Khamlichi et al. 1999, 2000; Roitsch and Gonzáles 2004). Our revised model (Figure 7) implies that prior to the induction of the *Mn1* gene, ~8 DAP in *Mn1* (Cheng et al. 1996), growth of caryopses of both genotypes might involve only the pathway in which cytokinins regulate G1/S of the cell cycle. Note that cytokinins and sucrose induce D-type cyclin CycD3 expression, and additionally, sucrose alone induces CycD2 (Riou-Khamlichi et al. 1999, 2000). The net result is a similar number of cells in *Mn1* and *mn1* caryopsis at that stage of development (Figures 1, 2). However, soon after the *Mn1* gene is induced, after 8 DAP (Cheng et al. 1996), possibly by cytokinins (Figure 7), an additional pathway might be implied in *Mn1* caryopsis, resulting in enhanced sink strength for photosynthates to the developing endosperm and embryo. The model then predicts that, together with the second pathway, at this time additionally stimulated by glucose (Figure 7) (Riou-Khamlichi et al. 2000), both cell divisions and sink metabolism participate in the growth of *Mn1* caryopsis. A reduction of cytokinin concentration at 12 DAP and later might be related to the subsequent decreasing transcript level of the *Mn1* gene (Chourey et al. 2006) and corresponding lower enzyme activity of INCW2 (Cheng et al. 1996). Therefore, according to the model (Figure 7), the residual level of cytokinins would be sufficient for the induction of ~60% of INCW2 activity determined between 16 and 20 DAP (Cheng et al. 1996) and for maintaining cell divisions in the sub-aleurone layer of the caryopsis (Figure 1). In contrast with the *Mn1*, *mn1* caryopsis contained higher concentrations of cytokinins, with a higher proportion of their metabolically active forms (Figure 6). The higher total amount of cytokinins and higher ratio of active cytokinins, together with $<$ 2% cell wall invertase enzyme activity contributed by the *Incw1* gene (Chourey et al. 2006), may partially compensate the absence of the INCW2 enzyme in regulating CycD3 and CycD2 induced cell divisions by cytokinins and sucrose in later stages of the *mn1* endosperm. In spite of the proposed D-cyclin pathway in the *mn1* caryopsis (Figure 7), the *mn1* endosperm showed only 55% of the cells at 16 DAP and only a quarter the volume of the wild type endosperm (Vilhar et al. 2002). According to the model this might be a consequence of the INCW2-deficiency (Cheng et al. 1996) and associated traits such as shorter cell division phase (Figure 3) and lower sink strength (Figure 7).

Materials and Methods

Plant material

Maize (*Zea mays* L.) plants of *Mn1* and the *mn1* reference allele in the W22 inbred line were grown at the experimental farms of the University of Florida, Gainesville, FL, USA during the summer of 2005 and 2006. Developing kernels from hand-pollinated plants were harvested at 6, 8, 12, 16 and 20 d after pollination (DAP). Caryopses were individually excised from the ear, taking care to include the pedicelar region, and fixed for cytological studies or immediately frozen in liquid nitrogen for cytokinin analysis. Frozen samples were stored at −80°C until cytokinin analysis. Prior to analysis, the caryopses were lyophilized. Caryopsis of the 6 DAP stage was used whole. The others were divided to the upper and basal parts with a razor blade just above the pedicel. Basal sections included maternal tissues, pedicel and pericarp and the filial basal endosperm transfer layer (Figure 1). In caryopses older than 8 DAP this section comprised the sucrose turnover region. Besides pericarp and developing endosperm, the upper sections of 8 DAP stages also included a large portion of maternal nucellar tissue, whereas endosperm and surrounding pericarp were predominantly present in the older stages. There, the upper section represented the storage region of the developing caryopsis.

Number of cells in the endosperm

The total number of cells in the endosperm was estimated using a 3-D model described by Vilhar et al. (2002). Replicates were measured in unstained Paraplast Plus (Sherwood Medical, St. Louis, MO, USA) embedded median longitudinal caryopsis sections.

Absolute growth rate was estimated from cell numbers data as a first derivative of the Richards' growth equation (Richards 1959) in modified notation (Goudriaan and van Laar 1994):

$$N = \frac{N_{\max}}{\left(1 + ve^{-k(t-t_m)}\right)^{1/v}} \qquad (1)$$

The growth function (1) was fitted to the experimental data and derived in GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). N is the number of cells at time t, whereas N_{max} (maximum number of cells), t_m (the time when absolute growth rate is maximal) and parameters k and v were determined from the fit. The R^2 value (goodness of fit) was 0.97 for *Mn1* and 0.92 for *mn1* endosperm cell number data.

Mean cell doubling time (MCDT) was calculated from:

$$MCDT = \frac{\ln(2)}{\frac{\ln\left(N_{t_2}/N_{t_1}\right)}{t_2 - t_1}} \qquad (2)$$

MCDT is an estimate for the time needed to double the cell population if the growth is exponential; N_{t1} and N_{t2} are the number of cells at times t_1 and t_2, respectively.

Endosperm growth is close to exponential in the initial phase until the absolute growth rate reaches a maximum [t_m, eqn. (1)]; later the MCDT increases rapidly.

Nuclear DNA content and cell volume measurement

Cytological parameters of the developing maize caryopses were measured in median longitudinal tissue sections (12–20 μm thick) of caryopses fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% glacial acetic acid) and embedded in Paraplast Plus (Sherwood Medical). Relative nuclear DNA amount was measured as integrated optical density in Feulgen-stained tissue sections using image densitometry with the interphase-peak method as described in Vilhar et al. (2001) and adapted for use with tissue sections (Vilhar et al. 2002). Nuclear volume was estimated as a sphere based on the nuclear area in the images (Kladnik et al. 2006). The amount of nuclear DNA was expressed in C-value units (1C represents the nuclear DNA content of a non-replicated haploid genome).

Cell volume was measured in the same tissue sections using autofluorescence of cell walls, essentially as described before (Vilhar et al. 2002) by outlining the cell walls visible due to their autofluorescence on UV excitation and estimating the cell volume as a sphere.

Cytokinin analysis

Developing kernels were frozen in liquid nitrogen immediately after harvest and then lyophilized. Cytokinins were analyzed as described by Salopek-Sondi et al. (2002). Dry plant material was ground with an Ultra Turrax-25 basic blender (IKA-Werke, Germany) in cold (4 °C) 80% aqueous methanol. The extraction of cytokinins, from 100 mg of ground plant material on a rotary shaker in cold 80% methanol for 1.5 h at 4 °C, was repeated three times. Collected extraction fractions were filtered, dried with a rotary evaporator (Heidolph, Germany) at 40 °C, re-suspended in 3 mL of 1 M formic acid and passed through a polyvinylpolypyrrolidone (Sigma-Aldrich, St. Louis, MO, USA) column to reduce the content of phenols and quinones, and again dried with a rotary evaporator at 40 °C. Samples re-suspended in 100 μL 70% ethanol and 900 μL phosphate-buffered saline (PBS) (pH = 7) were applied to a pre-immune column (OlChemim, Olomouc, Czech Republic) and finally to immunoaffinity columns (OlChemim) with bound polyclonal antibodies against isoprenoid or aromatic cytokinin metabolites. Bound cytokinin metabolites were eluted with 3 mL of cold methanol (−20 °C), immediately dried with a rotary evaporator at 40 °C and re-suspended in 5% methanol. Samples were analyzed by high-performance liquid chromatography (HPLC) using a Nova Pack C_{18} column (4 μm spherical particles, 150 mm × 3.9 mm) (Waters, Milford, MA, USA) equilibrated with 1 mM aqueous triethylamine acetate (pH 7) (solvent A), containing a 5% mixture of methanol-acetonitrile (1:1, v/v) (solvent B). The separation and elution program was as follows: a gradient elution from 0 to 30 min (5 to 20% solvent B) from 30 to 35 min isocratic elution (20% solvent B), a gradient elution from 35 to 40 min (20 to 30% solvent B) and isocratic elution from 40 to 50 min (30% solvent B). The effluent was passed through a Waters 996 photodiode array spectrophotometer and 1 mL fractions were collected. Cytokinin metabolites were identified on the basis of retention times and spectral properties of a set of standards: *trans*-zeatin, Z; dihydrozeatin, DHZ; N^6-isopentenyl adenine, iP; *trans*-zeatin riboside, ZR; dihydrozeatin riboside, DHZR; N^6-isopentenyl adenosine, iPA; *trans*-zeatin-9-glucoside, Z-9-G; dihydrozeatin-9-β-D-glucoside, DHZ-9-G; N^6-isopentenyladeneine-9-glucoside iP-9-G; *meta*-topolin, mT; *ortho*-topolin, oT; *meta*-topolin riboside, mTR; *ortho*-topolin riboside, oTR; N^6-benzyladeneine, BAP (all from OlChemim). They were quantified by integrating the areas under the peaks measured at 265 xnm and comparing them with integrated areas of peaks of known standard quantities. The efficiency of the isolation method was estimated by running known quantities of standards through the extraction, isolation and quantification procedure, either alone or added to the sample. Recoveries for each isoprenoid cytokinin species were as follows: Z (38–40%), DHZ (30–36%), iP (2–4%), ZR (58–59%), DHZR (52–56%), iPA (22–24%), Z-9-G (59–61%), DHZ-9-G (45–50%), iP-9-G (24–28%). Corrections were made for the losses. No aromatic cytokinins corresponding to the added standards were detected in the examined samples.

The combined results showed that the period between 8 and 12 DAP is critical for further caryopsis development, so additional samples from 8- and 12 DAP-old caryopses were analyzed in more detail in two growing seasons.

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